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14. ABSTRACT

The research covered under this award is designed to further study our original finding that the epigenetic drug 5-azacytidine increases the responsiveness of prostate cancer cells and xenografts to radiation therapy by impairment of DNA double strand break repair. One of the most important goals of the project is to examine the efficacy of a combined regimen of 5-azacytidine, androgen ablation therapy, and radiation for treatment of advanced prostate cancers. We here present our second annual report, in which we demonstrate that combining 5-azacytidine with the two standard of care modalities for prostate cancer (androgen ablation and radiation) results in a temporary but complete control of tumor progression in a mouse model utilizing androgen-independent human-derived prostate cancer xenografts. Importantly, a single administration of the experimental regimen halted tumor progression for a period of a month, whereas tumors treated with radiation alone rapidly progressed during that period. Although tumors eventually resumed progression, the overall l time needed for triplication of the median volume was doubled as compared to the tumors treated with radiation alone. We expect that administration of repeat doses at the time of relapse, will further improve the overall outcome of the treatment, and we intend to further examine this hypothesis. We conclude that our proposed novel combination therapy of 5-azacytidine, androgen ablation, and radiation is indeed markedly more effective than the single modalities. These findings solidify the clinical potential of 5-azacytidine for treatment of advanced prostate cancers. In addition, our findings give further credibility to earlier observations that 5-azacytidine re-sensitizes advanced androgen independent tumors to andogen ablation therapy, thereby significantly expanding the applicability of this type of treatment. In the third year of the project, we aim to examine the effects of our treatment regimen on the metastatic potential of prostate tumors.

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Introduction

As a component of our ongoing research on DNA double-strand break (DSB) repair mechanisms and their applicability to the improvement of radiation therapy, we have previously discovered a radiation-sensitizing effect of the epigenetic drug Vidaza (5-azacytidine) on the androgen-independent prostate cancer lines PC-3 and DU-145 and on PC-3 based xenografts. Thus far, we have been able to demonstrate that this radiation- sensitizing effect of 5-azacytidine is at least in part due to a clear suppression of the DSB repair pathway non-homologous end-joining (NHEJ). The project funded by this award is geared towards two main goals: 1) the elucidation of the pathways involved in 5-azacytidine mediated radiation-sensitization of prostate cancer cells, and 2) the examination of the effectiveness of combing a 5-azacytidine/radiation protocol with androgen ablation on suppression of xenograft tumor growth. We here present the second annual report on our progress.

Body

Short background

Prostate cancer is the most common cancer in men in the US. Approximately 190.000 new cases are reported annually. Although radical prostatectomy is a first line of treatment, this methodology has many emotionally burdening side effects, is not always elected, and is not always successful in removal of all cancerous tissue. The most commonly used alternative treatment modalities include radiation and androgen deprivation, which are both aimed at slowing down progression of the tumor. Unfortunately, a significant percentage of patients receiving androgen ablation therapy eventually develop resistance against this treatment and progress into androgen-independent growth and, eventually, the onset of metastasis.

It has been shown by other research groups that the drug 5-azacytidine – currently FDA approved for the treatment of myelodysplastic malignancies – restores responsiveness of androgen-insensitive prostate tumors to androgen ablation therapy, even at relatively low doses (1-4). This finding has triggered a strong and contemporary interest in the use of 5-azacytidine as a treatment modality for prostate cancers, possibly administered on a long-term basis in an early stage of the disease, in order to delay or avoid the onset of androgen-insensitivity.

As a result of our own research efforts on DNA repair, we established a hitherto unknown ability of 5-azacytidine to significantly suppress the repair of radiation-induced DSB's. Data supporting this finding has been previously disseminated to the DOD (technical reporting, award W81XWH-07-1-0543) and will be combined with the data presented in this report for publication (see Reportable Outcomes section). In short, we observed a statistically very significant delay in the repair of radiation-induced DSBs upon treatment of prostate cancer cells with 5-azacytidine, as evidenced by an approximately 4-fold higher level of residual gamma-H2AX foci in 5-azacytidine treated cells versus untreated cells. In

addition, we examined the proficiency of the main DSB repair pathway – NHEJ – by measuring the overall efficiency of artificially introduced DSB substrates, and found a 50-55% reduction upon treatment with 5-azacytidine. Finally, we observed a 30-plus day delay in progression of PC-3 based xenograft tumors in nude mice upon dual exposure to radiation and 5-azacytidine. Clearly, these combined finding strongly argue for a radiation-sensitizing potential of 5-azacytidine, at least in part mediated through the suppression of DSB repair via the NHEJ pathway.

Combining the above discussed abilities of 5-azacytidine to potentiate the effects of the two major treatment modalities for prostate cancers – radiation and androgen ablation – could theoretically be a very powerful approach to markedly delay or even halt progression of early stage prostate cancers into the later stages. The facts that 1) 5-azacytidine appears to display its potentiating effects at low concentrations and 2) is an already FDA approved compound, will drastically enhance the practicability of actually implementing a combined 5-azacytidine, radiation, and androgen ablation protocol in a clinical setting.

The project covered under this award has two principal aims. The first aim involves examination of the effects of 5-azacytidine on expression levels and epigenetic regulation of general DNA repair enzymes, as well as specific DSB and/or NHEJ enzymes. Accomplishments regarding this first aim have been extensively discussed in our first annual report and will be summarized in the results section below. The second aim revolves around assessment of the feasibility of combining the prostate cancer treatment modalities 5-azacytidine, radiation and androgen ablation in a single protocol and to quantify the influence of this protocol on tumor size and on the onset of metastasis. In keeping with the approved Statement of Work (SOW), we have booked considerable progress this year with respect to the second aim, which we will present in the sections below.

Short summary of research accomplishments in the first year

As reported in more detail in our previous annual report (Feb 27, 2012), in the first year of the project we have directed our efforts towards a further elucidation of the molecular mechanism by which 5-azacytidine influences NHEJ. Following the approved SOW for aim 1, we have executed this line of research by analyzing changes in steady state mRNA levels of NHEJ involved genes and general DSB repair genes in prostate cancer cells as an effect of 5-azacytidine and/or radiation exposure. We also analyzed 5-azacytidine/radiation induced epigenetic changes in the cytosine methylation status of several routinely analyzed DSB repair genes (but not in the NHEJ involved genes yet). Finally, we analyzed the modulation of expression levels of regulatory miRNA species in prostate cancer cells as a result of 5-azacytidine and/or radiation exposure. By following this methodical approach, we were able to examine the effect of 5-azacytidine in prostate cancer cells on a transcriptional, epigenetic, and regulatory level.

The obtained results indicated that 5-azacytidine is not altogether likely to directly influence the expression and/or activity of the core NHEJ factors, but rather modulates the signaling pathway upstream of the NHEJ pathway. This likely involves alteration of the ATM/ATR activated signal transduction cascade which is initiated upon introduction of DSB's. Part of the inhibitory activity of 5-azacytidine on overall DSB repair may also be due to modulation of factors directly involved in the secondary DSB repair pathway Homologous Recombination (HR). In addition, we found a marked elevation of the regulatory miRNA miR-155, which might be involved in post-translational modification of NHEJ factors. In conclusion, our results indicated that 5-azacytidine likely interacts with the DSB repair machinery on 3 levels: 1) indirect modification of NHEJ by interference with the upstream signal transduction cascade, 2) direct modification of HR by direct interference with several HR core factors, and 3) post-translational modification of DSB repair by upregulation of regulatory RNA species.

Research accomplishments in the second year

In the second year, we have successfully accomplished virtually all research goals outlined in last year's annual report, with only a minor delay in aim 1B. Although we have re-located our research from the Nevada Cancer Institute to the University of Arizona at the beginning of the second project year, we projected that we would be able to complete aims 1 and 2A, which entailed 1) finalization of the experiments needed to establish a working model for the DSB repair inhibitory activity of 5-azacytidine, and 2) determining the efficacy of a triple combination therapy of 5-azacytidine, flutamide, and radiation on human prostate cancer xenografts. Especially this latter endeavor has yielded very exciting and publishable novel data, clearly demonstrating the validity of a combination regimen of 5-azacytidine, radiation, and androgen deprivation for the treatment of prostate cancers. In addition, we have already advanced into aim 2B, slightly ahead of schedule.

Aims 2A and 2B

A most important outcome of our efforts in this second year of the project is the completion of Aim 2B of the approved SOW in its totality. This aim entailed 1) verification of the radiation-sensitizing potential of 5-azacytidine for prostate cancer xenografts, and 2) examination of the efficacy of a novel combination regimen of androgen ablation, 5-azacytidine administration, and radiation. As discussed earlier in this report, 5-azacytidine has been demonstrated to re-sensitize androgen insensitive prostate cancer cells to androgen ablation therapy (1-4) as well as to radiation treatment (our own observations) and therefore has the potential to be a potentiator for both treatment modalities. We here demonstrate that this is indeed the case for human derived androgen-insensitive PC-3 xenografts when treated with the canonical androgen repressor flutamide and concurrent X-ray irradiation.

In order to arrive at this result, we subcutaneously injected 5 groups of male athymic nude NCr Nu/Nu mice (16 animals per group) with approximately 4.10^6 PC-3 cells. No matrigel was used for this inoculation. The injections resulted in formation of PC-3 based xenografts on the flank of the animals. When most of the xenograft tumors reached an average volume of 500 mm3 at the 25^{th} day post injection, the 5 groups were treated as follows (see figure 1): (1) control group, (2) 5-azacytidine treatment alone, (3) flutamide treatment alone, (4) radiation treatment alone, (5) combination treatment with 5-azacytidine, flutamide, and radiation.

Flutamide treatment, when applicable, consisted of subcutaneous implantation of a commercially available slow-release pellet (Innovative Research of America, Cat # SA-152 25 mg/pellet 60 day release) resulting in a 50 mg/kg accumulative dose over 60 days. This pellet was implanted 2 days before the start of 5-azacytidine and radiation treatments (see figure 1). Groups that did not receive flutamide treatment received an inert placebo pellet (Innovative Research of America Cat# SC-111) instead. Vidaza (5-azacytidine) was administered by intravenous injection following a 2.5 mg/kg/day dosing schedule for 5 consecutive days. Animals not treated with 5-azacytidine received a placebo injection consisting of saline. Radiation treatment, when applicable, was delivered with a Cobalt-60 teletherapy unit and given concurrent with 5-azacytidine (or saline) administration, following a delivery schedule of 2.5 Gy/day for 5 consecutive days. In order to deliver radiation dose localized to the tumor, animals were placed within a custom made shielding tube, which allowed protrusion (and therefore irradiation) of the xenograft tumor. Tumor volumes were recorded during the treatment period and during a 45 day period after treatment, in which no further treatment of any kind was rendered. The results are plotted in figure 1.

As expected, administration of 5-azacytidine or flutamide as single modalities did not result in significant deviation of the tumor growth characteristics from the control group, indicating that neither modality by itself was successful in controlling tumor progression (figure 1). Localized delivery of radiation to the tumors did result in modest reduction of tumor volumes, but tumor progression was never halted and therefore tumor control was never obtained with radiation delivery as a single modality under the chosen conditions. Tumor volumes in the radiation single modality group reached the same level as the tumor volumes in the control and 5-azacytidine and flutamide single modality group at approximately 30 days post treatment. However, this effect is at least caused by the fact that around the 25 day time point several animals in de control and single modality groups had to be euthanized for humane reasons after an approximate tumor volume of 1750 mm^3 was observed. Therefore, these graphs curve towards a plateau and the fact that the curve for the radiation group reaches the same plateau does not indicate that radiation single modality treatment ceased to reduce tumor growth rates after the 30 day post treatment mark. For this reason, our data are best interpreted before the 35 days post treatment point. (Raw data are compiled in appendix 1)

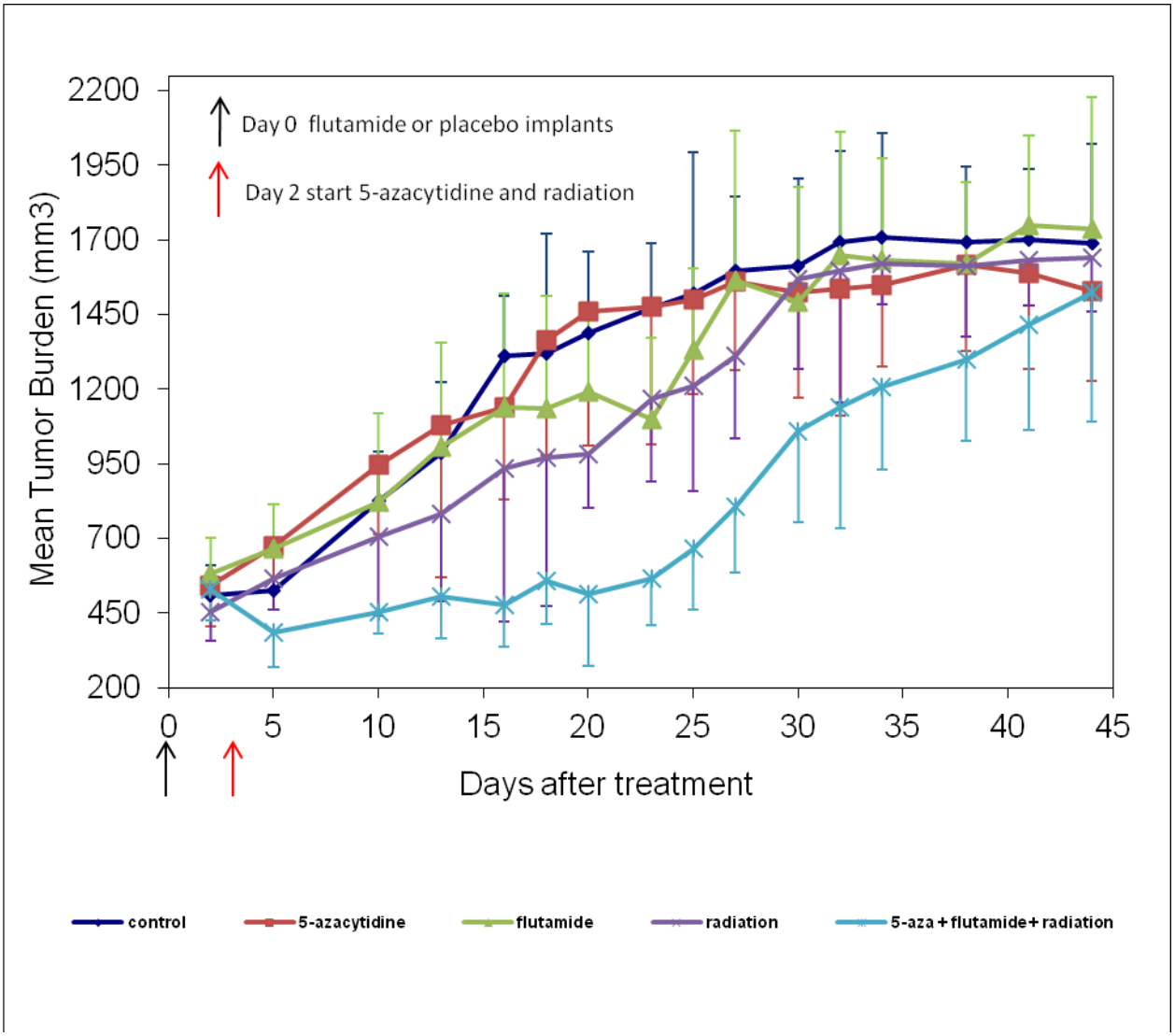


Figure 1. *Progression of PC-3 based xenograft tumor volumes in athymic nude mice after treatment with single modalities (5-azacytidine, flutamide, and radiation) or with a combined regimen of 5-azacytidine, flutamide, and radiation. Each treatment group consists of 16 animals.*

Importantly, we observed complete control of tumor volume progression in the first 25 days post treatment in the triple modality group, treated with a combination of flutamide, 5-azacytidine, and radiation (figure 1). During this period, the median tumor volume did not significantly increase beyond the initial 500 mm^3 volume that was present pre-treatment, in sharp contrast to the control and single modality groups in which the tumor burden rapidly increased during this period. After the 25 day period of complete tumor control, a relapse in tumor control was observed and tumor volumes started

increasing again. However, it did take an additional 20 days past the relapse point for tumors to reach the 1750 mm3 cut-off mark where the animals were euthanized.

Based on these observation we conclude that 1) complete control of PC-3 based xenograft tumors can be achieved during a period of approximately a month after an initial administration of a triple modality treatment consisting of 5-azacytidine, flutamide, and radiation treatment, and that 2) the time needed for a triplication of tumor volume is almost doubled as a result of a single administration of this triple modality. This result is quite remarkable and most definitely opens windows for the future application of our proposed combination therapy in a clinical setting.

In view of the fact that tumor growth was practically abolished for the duration of a full month before relapse occurred, we suggest that a repeat administration of 5-azacytidine and radiation around the time of the relapse (flutamide is continuously released by the implanted pellets), is likely to markedly improve the outcomes even further. We fully expect that a repeat treatment would further delay the onset of relapse and further expend the time needed for tumor progression. If remaining time and funding on this project allow, we will commit to verifying this hypothesis by repeating the experiment and including repeat treatment points. We are, however, giving priority to the fulfillment of the second part of aim 2, in which we will examine the metastatic potential of prostate xenograft tumors after triple modality treatment (see discussion below).

When translating the above discussed results to a clinical setting, we must keep in mind that the disease progression in human patients is actually very slow, in contrast to the rapid and enhanced tumor progression observed in the xenograft model. Although useful as a tool to study a normally slowly evolving disease in a manageable timeframe, the limitations of the xenograft models utilized by us make it likely that treatment regimens for patients will differ considerably from those presented here. Most likely, repeated administration of the treatment modalities will be necessary in order to maintain tumor control over extended time spans.

Nevertheless, our finding constitute a solid proof of principle for the validity of our hypothesis that 5-azacytidine can potentiate the current standard of care treatment of prostate cancers by means of androgen ablation and radiation. Although somewhat outside the scope of our study, the presented data on androgen insensitive PC-3 xenografts also give further credibility to the notion that 5-azacytidine may re-sensitize androgen insensitive prostate cancer cells back to androgen sensitivity (1-4). Although flutamide has been historically used to delay transition of androgen sensitive disease into advanced androgen insensitive disease, the use of flutamide as a single modality ceases to be effective upon entering the androgen insensitive stage. However, in combination with 5-azacytidine, the use of flutamide might very well be useful even in the context of an advanced disease.

In addition to the work discussed above, we have also started working on the execution of aim 2B of the project. As stated in the approved SOW, this entails a study into the metastatic potential of

TRAMP-C2 mouse prostate adenocarcinoma based xenografts after administration of the triple modality (5-azacytidine, flutamide, and radiation.

In order to monitor the onset of metastasis, it will be necessary to tag the TRAMP-C2 cells with a fluorescent label which allows for identification of metastases in target organs. Therefore, we have created a stable transgenic cell line by transfecting a TRAMP-C2 culture directly obtained from the American Type Culture Collection (ATCC, item number CRL-2731) with the mammalian Green Fluorescent Protein (GFP) expression vector pEGFP-N1, utilizing lipofectamine as a delivery agent. After selection on neomycin and several rounds of subculturing, we obtained a dozen TRAMP-C2-GFP clones which stably expressed the GFP protein throughout replication (figure 2).

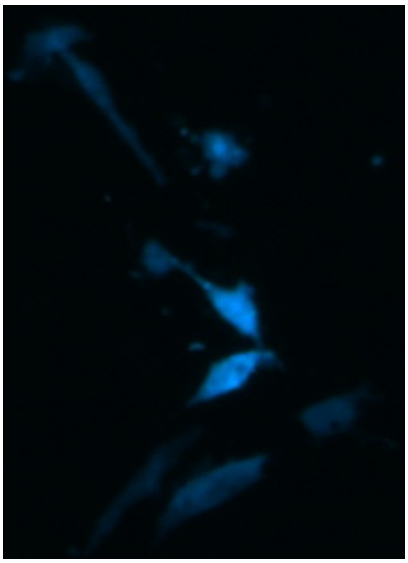


Figure 2*. Stably transfected TRAMP-C2-GFP cells. These cells are currently used to introduce fluorescent xenograft tumors in C57BL/6J mice.*

We are currently in the process of subcutaneously injecting the two best expressing clones of our stable TRAMP-C2-GFP cell line in a small sample group of 10 male C57BL/6J mice in a manner identical to the methodology applied for experiment 2A. This scouting experiment will determine the following experimental parameters which will be utilized to optimize the experimental set-up of the definitive experiment: (1) solidify the choice of TRAMP-C2-GFP clone best suited for detection of a GFP signal, and (2) the failure rate of tumor formation in the combination TRAMP-C2-GFP cells and C57Bl/6J mice.

Based on our observations during execution of experiment 2A (the failure rate of tumor formation and the standard deviations in recorded tumor volumes) we are currently projecting our experimental

set-up to be as follows: we will create 5 treatment groups identical to those in experiment 1A, each group will consist of 15 mice. At 3 time points (20, 40, and 60 days past treatment) 5 animals from each group will be sacrificed, after which histological examination of the primary organs by fluorescence microscopy will be performed in order to scan for the presence of GFP expressing metastases.

Aim 1

Although we have previously demonstrated that 5-azacytidine introduces radiation sensitization of prostate cancer cells and xenografts by modulation of NHEJ-mediated DNA break repair, the experiments performed in year 1 of this project have not yielded evidence for a *direct* effect of 5-azacytidine on expression of the most common DSB repair and NHEJ genes, but rather for an as of yet unidentified effect upstream of the NHEJ pathway. However, it is still conceivable that 5-azacytidine modulates the cytosine methylation status of the NHEJ core genes, which were not included in the commercial panel analyzed in year 1. In order to address this issue, we have isolated the genomic DNA of human prostate cancer cells and a normal prostate epithelial control cell line and initiated an experiment designed to quantify the promoter CpG cytosine methylation status of every known core NHEJ gene (Ku70, KU80, DNA-PKCS, XRCC4, Ligase IV, and XLF).

For this experiment, which corresponds to aim 1B of the approved SOW, we treated 4 different prostate cell lines (PC-3, DU-145, LNCaP, and the control line PCS-440-010) with 6 different modalities: 1) control, 2) 4 Gy radiation, 3) 1 uM 5-azacytidine, 4) 10 uM 5-azacytidine, 5) 1 uM 5-azacytidine + 4 Gy radiation, 6) 10 uM 5-azacytidine + 4 Gy radiation. Cell cultures treated with 5-azacytidine received a fresh dose of 5-azacytidine every day, for a period of 3 days. Radiation was delivered by means of a Cobalt-60 teletherapy unit on the third day of the procedure. Triplicate biological repeats of each treatment group were performed. Genomic DNA was extracted from the cell cultures 1-2 hrs post irradiation. Genomic DNA of biological repeats were pooled in equimolar amounts into one sample. Therefore, we obtained 6 samples (6 treatment groups) of each cell line, totaling 24 samples.

At the time this report is written, these samples are being analyzed for promoter CpG cytosine methylation status of the Ku70, KU80, DNA-PKCS, XRCC4, Ligase IV, and XLF core NHEJ genes, as well as the ATM, ATR, Rad 51, and RAd 52 DSB repair genes. Analysis was performed by state of the art massARRAY technology. Although slightly different in design than the originally proposed pyrosequencing technique, this methodology is superior in sensitivity and accuracy and recently became available at the University of Arizona Cancer Center, thereby providing us with a unique and cost-effective opportunity to accommodate our research needs. In short, sodium bisulfite-treated genomic DNA was prepared according to the standard protocol of the Zymo Research company. Sodium bisulfite-treated DNA (5 ng) was seeded into a region-specific PCR incorporating a T7 RNA

polymerase sequence as described by the manufacturer (Sequenom). Primer sequences for the above mentioned genes were designed using EpiDesigner 6. The resultant PCR product was then subjected to in vitro transcription and RNase A cleavage using the MassCLEAVE T-only kit, spotted onto a Spectro CHIP array, and analyzed using the MassARRAY Compact System matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer (Sequenom). Each sodium bisulfite-treated DNA sample is being processed in two independent experiments. Data are analyzed using Sequonom's EpiTyper software.

At the time of submission of this report, the above described massARRAY data was still in the process of generation and compilation. Although we anticipated to completely finish the experiment before the deadline of this report, several minor delays including initial calibration issues of the massARRAY and a change in personnel (senior research specialist Mr Steven Brotman was replaced by Mr Alfred Gallegos), made it impossible to include the final data in this report. We will therefore have to include the results in our next progress report.

Projections for the third year

In this second year of the project we have re-located our research from the Nevada Cancer Institute to the University of Arizona, Department of Radiation Oncology. Although some minor delays in the finalization of Aim 1 were encountered as a result of the move, we have successfully managed to maintain current with the projected flow of experiments as presented in our first annual report. Therefore, the projection for the third year will remain unaltered.

We expect that data generation and compilation as needed for completion of aim 1B will be finished by April 2013, without causing any delay in other parts of the overall project. The further execution of aim 2B (analysis of metastatic potential of prostate cancer cells after treatment with radiation, androgen ablation, and 5-azacytidine) is expected to fit the following time line:

March - April 2013: Small scale test run to verify the success/failure rate of xenograft tumor formation from GFP-TRAMP-C2 cells, as well as selection of the most suitable GFP-TRAMP-C2 clone.

May – August 2013: Implantation of GFP-TRAMP-C2, followed by a xenograft tumor formation and commencement of single modality or combination treatments. Collection of tissue samples from primary tumor, lymph nodes and primary organs at set time points. At this time a decision will also be made whether a repeat experiment will be necessary.

September 2013 – February 2014: Histological examination of the collected tissue samples, measurements of tumor progression, data analysis, statistical analysis, and optimization.

Therefore, at present we expect to be able to fully complete the project within the time frame originally set forth. In addition, we would like to repeat the experiment outlined under aim 2A with repeat doses of 5-azacytidine and radiation administered at the point(s) of tumor growth relapse, as discussed in the results section. However, time and available funding will be directed with priority to the fulfillment of aim 2B, as stipulated in the original SOW.

Key Research Accomplishments up to present (years 1 and 2)

1) Established that 5-azacytidine likely decreases DSB repair by a) modulation of the signaling cascade upstream of the NHEJ pathway and b) modulation of factors directly involved in HR.

2) Established that in addition to direct regulation of the NHEJ and HR pathways, 5-azacytidine may possibly interact with DSB repair by markedly elevating the micro-RNA species miR-155.

3) Established that a previous clinical observation of increased responsiveness of prostate tumors to 5-azacytidine exposure may not be mediated by modulation of expression of the androgen receptor.

4) Established that a combination therapy consisting of 5-azacytidine administration, androgen ablation therapy by flutamide administration, and local irradiation of the tumor, results in a very significant delay of tumor progression compared to the single modality treatments. Complete control of tumor progression was observed for a period of a month after administration of a single treatment regimen.

5) Established further proof for the notion that flutamide treatment in combination with 5-azacytidine (and radiation) may be beneficial in a clinical setting even after progression of the disease into an androgen-insensitive status.

Reportable Outcomes

Manuscript ‘Low-dose 5-azacytidine impairs DNA double strand break repair through non-homologous end-joining’ by Eric Weterings, Pamela Dino, James Symanowski, and Giuseppe Pizzorno. This manuscript is completed, but may ultimately be consolidated into the following manuscript under preparation:

Manuscript ‘5-azacytidine sensitizes androgen insensitive prostate cancer xenografts to radiation and androgen ablation therapy’ by Gillian Paine, Alfred Gallegos, and Eric Weterings. This manuscript is currently in progress and will ultimately include the data that will likely be obtained in the year.

Conclusion

In this second annual report, we have presented convincing evidence that androgen insensitive prostate cancer xenograft tumors can be significantly sensitized to radiation and androgen ablation (flutamide) therapy by administration of the epigenetic drug 5-azacytidine. Importantly, full control of tumor progression (no increase in median tumor volume) was established for the period of a month with only one initial regimen of a low, non-toxic dose of 5-azacytidine. Although eventually the xenograft tumors did resume progression, it lies within reason to expect that a strategically timed repeat dose of 5-azacytidine and radiation will further extend the period in which tumor control is observed.

These data do not only provide a solid proof-of-principle for our notion that 5-azacytidine has radiation-sensitizing properties, but opens vista's for inclusion of 5-azacytidine into clinical protocols for advanced prostate cancers. Our results give credibility to earlier published data, suggesting that 5-azacytidine can expand the use of flutamide therapy into the androgen-insensitive phase of prostate cancer, by (at least partially) re-sensitizing the tumor to androgen ablation.

Although our data present a very promising outlook on clinical utility, the limitations of a xenograft model must be kept in mind for interpretation and translation of our results to a human patient. Unlike the xenograft tumors we use, prostate cancer progression is generally slow which makes the need for repeat administration of 5-azacytidine and radiation in a clinical setting highly likely. However, we have observed significant radiation sensitization with only low levels of 5-azacytidine which were well tolerated by our test animals and at least an order of magnitude below the maximum tolerated dose. If this finding translates into the clinic, the risk of repeat administration of this drug should not outweigh the benefits.

On a mechanistic level, we have thus far been able to determine that 5-azacytidine is capable of increasing the response of prostate to radiation therapy by repressing the ability of prostate cancer cells to repair radiation induced DNA breaks. This is likely accomplished on at least three levels: 1) by modulation of the signaling pathway that initiates the NHEJ pathway, 2) by direct modulation of some of the components of the HR pathway, and 3) on a post-translational level by increasing the expression of several regulatory RNA species.

Now the clear benefits of the use of 5-azacytidine as a radiation sensitizer for the control of primary xenograft prostate tumors has been established, it will be of high importance to study the effects of this powerful combination treatment on the metastatic potential of the tumor. This will be the main focus of our research in the third and final year of this project.

References

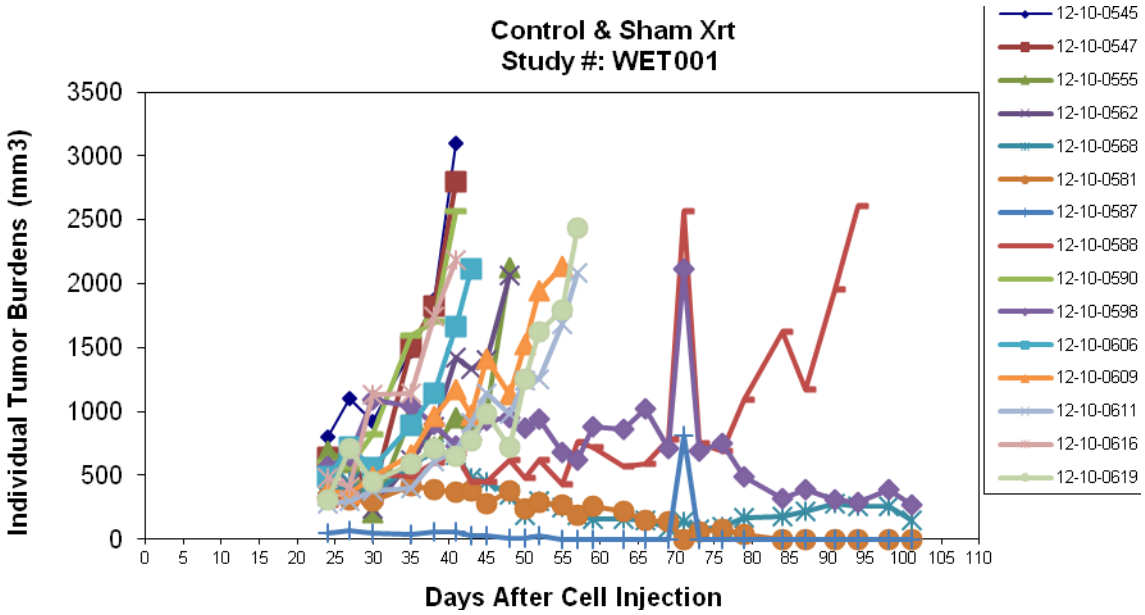
1. Sonpavde G, Aparicio A, Guttierez I, et al. Phase II study of azacitidine to restore responsiveness of prostate cancer to hormonal therapy. Clin Genitourin Cancer 2007;5:457-9.

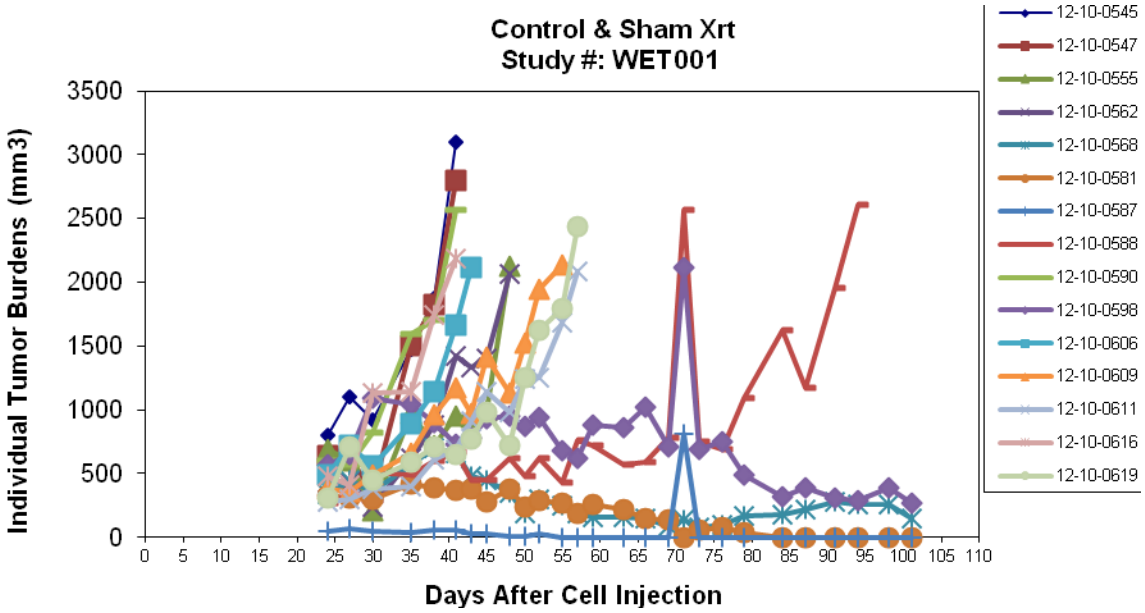
2. Sonpavde G, Aparicio AM, Zhan F, et al. Azacitidine favorably modulates PSA kinetics correlating with plasma DNA LINE-1 hypomethylation in men with chemonaive castration-resistant prostate cancer. Urol Oncol 2009.

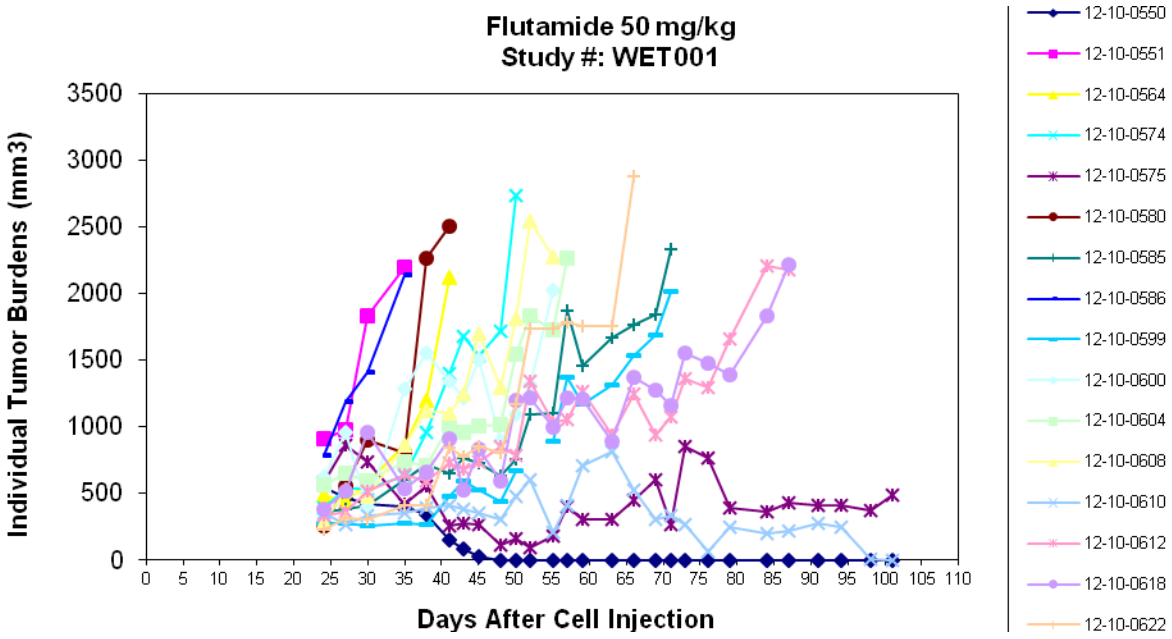
3. Zorn CS, Wojno KJ, McCabe MT, Kuefer R, Gschwend JE, Day ML. 5-aza-2'-deoxycytidine delays androgen-independent disease and improves survival in the transgenic adenocarcinoma of the mouse prostate mouse model of prostate cancer. Clin Cancer Res 2007;13:2136-43.

4. Gravina GL, Festuccia C, Millimaggi D, et al. Chronic azacitidine treatment results in differentiating effects, sensitizes against bicalutamide in androgen-independent prostate cancer cells. Prostate 2008;68:793-801.

Appendix 1, compilation of raw data associated with figure 1 (aim 2A).



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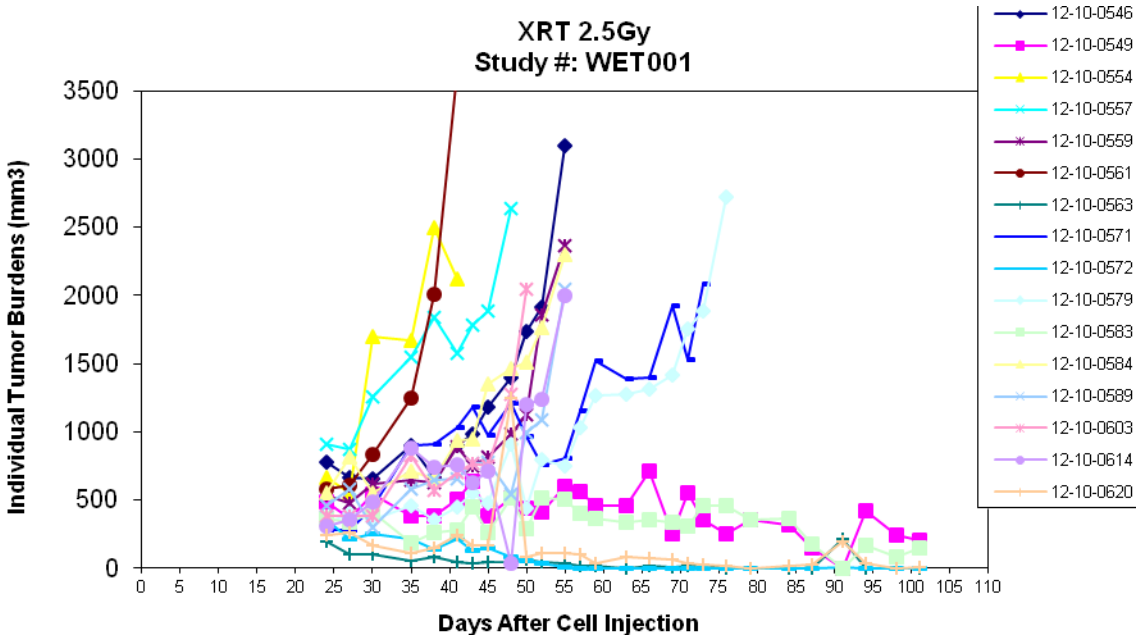
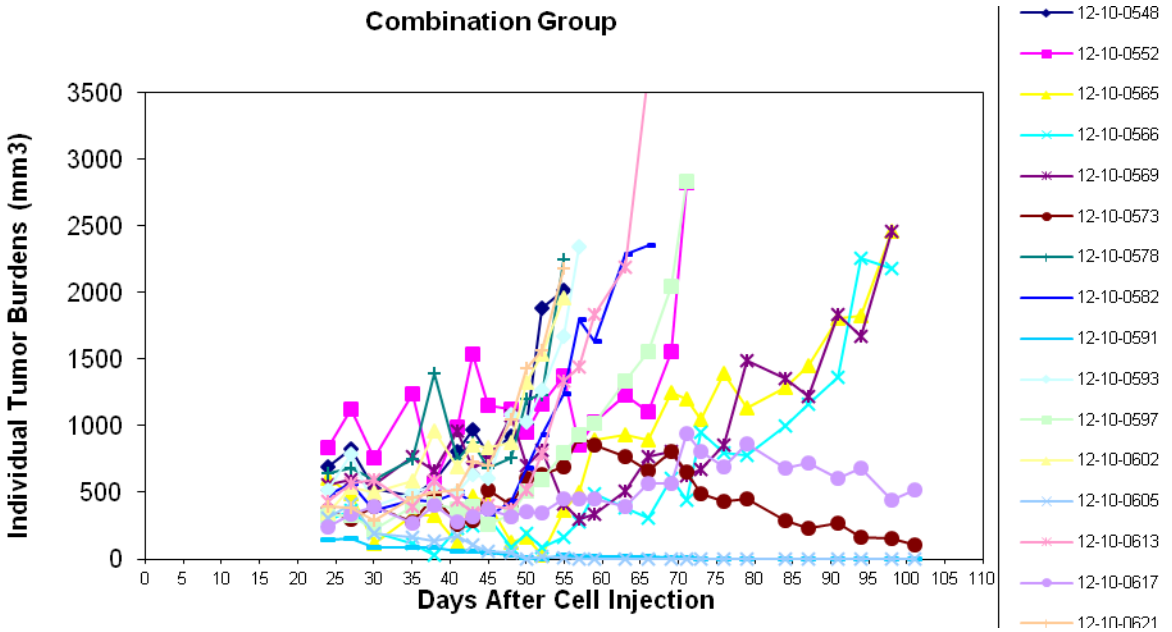
12-10-0575
12-10-0580
12-10-0585
12-10-0586
12-10-0599
12-10-0600
12-10-0604
12-10-0608
12-10-0610
12-10-0612
12-10-0618
12-10-0622


XRT 2.5Gy
Study #: WET001
Individual Tumor Burdens (mm3)
Days After Cell Injection
12-10-0546
12-10-0549
12-10-0554
12-10-0557
12-10-0559
12-10-0561
12-10-0563
12-10-0571
12-10-0572
12-10-0579
12-10-0583
12-10-0584
12-10-0589
12-10-0603
12-10-0614
12-10-0620


Combination Group
Individual Tumor Burdens (mm3)
3500
3000
2500
2000
1500
1000
500
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110
Days After Cell Injection
12-10-0548
12-10-0552
12-10-0565
12-10-0566
12-10-0569
12-10-0573
12-10-0578
12-10-0582
12-10-0591
12-10-0593
12-10-0597
12-10-0602
12-10-0605
12-10-0613
12-10-0617
12-10-0621